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Multiplex Ligation-Dependent Probe Amplification of Uveal Melanoma: Correlation with Metastatic Death

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Purpose. To evaluate multiplex ligation-dependent probe amplification (MLPA) of uveal melanoma as a predictive tool for metastatic death.

Methods. Uveal melanoma specimens of 73 patients treated between 1998 and 2000 were included. DNA samples were analyzed with MLPA evaluating 31 loci on chromosomes 1, 3, 6 and 8, and the results were correlated with metastatic death.

Results. The patients (27 women; 46 men) had a median age of 60.6 years and a median follow-up of 6.2 years. Metastatic death occurred in 28 patients, correlating most strongly with chromosome 3 losses and gains on 8q (Cox univariate analysis, $P < 0.001$). Chromosome 6, region p25, gains correlated with good survival (Cox univariate analysis, $P = 0.003$). Prediction of metastatic death was improved by considering equivocal chromosome 3 losses as abnormal and by taking account of multiple risk factors, such as 8q gains, tumor diameter, and histologic features indicative of high-grade malignancy.

Conclusions. MLPA analysis of uveal melanoma predicts metastatic death if statistically insignificant losses of chromosome 3 are considered together with gains in 8q as well as clinical stage and histologic grade of malignancy. (*Invest Ophthalmol Vis Sci.* 2009;50:3048–3055) DOI:10.1167/iovs.08-3165

Approximately 40% to 50% of uveal melanomas are fatal, usually as a result of metastatic disease, which almost always involves the liver.[1] Estimation of risk of metastatic death would help reassure patients with a good prognosis while enabling any screening for metastasis, systemic adjuvant therapy, and other special measures to be targeted at patients with a poor survival probability.

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The main risk factors for metastasis in uveal melanoma include large basal diameter (LBD) of the tumor, ciliary body involvement, extraocular spread, epithelioid cellularity, closed connective tissue loops, high mitotic rate, and certain karyotypic abnormalities, particularly those involving chromosomes 1, 3, 6, and 8.[2–10]

The most important chromosomal abnormality associated with metastatic death is monosomy 3, which usually involves the loss of an entire chromosome.[6] Gains in chromosome 8 also indicate a poor prognosis and can consist of trisomy 8, isochromosome 8q, and additions in the *C-MYC* region.[7] Metastatic mortality also correlates with 1p loss.[10] Conversely, 6p gains are associated with a good prognosis, these abnormalities usually being mutually exclusive with monosomy 3.[7,9]

Since 1999, we have offered cytogenetic analysis to all patients with uveal melanoma treated by local resection or enucleation, and we have used fluorescence in situ hybridization (FISH) for this purpose. An audit of 356 patients showed a high correlation between monosomy 3 and metastatic death; however, in the absence of monosomy 3, the actuarial 5-year rate of metastatic death ranged from 31% to 6%. depending respectively on whether or not there were gains in chromosome 8.[11] We presumed that this was because of partial deletions that were missed by FISH.[12,13] To address this problem, we developed an artificial neural network that estimated survival prognosis according to age, sex, clinical tumor stage, histologic grade of malignancy, and presence or absence of monosomy 3.[14] To some extent, this program compensated for missed chromosome 3 deletions by estimating the likelihood of these being present, according to the other risk factors present. Although the artificial neural network alleviated the problem caused by the low sensitivity of FISH, we decided to deploy more sensitive methods of genomic typing, which could be applied in routine clinical practice.

We therefore adopted multiplex ligation-dependent probe amplification (MLPA), which comprises a set of 43 probes, each hybridizing to a specific genomic sequence.[15] The probes have different molecular weights and lengths so that after amplification by polymerase chain reaction (PCR), they can be separated by gel electrophoresis and quantified in comparison with control sequences. We used the Salsa P027 (MRC-Holland, Amsterdam, The Netherlands), a kit specifically designed for uveal melanoma. The kit comprises 12 control probes and test probes directed at 7 loci on chromosome 1, 13 on chromosome 3, 6 on chromosome 6, and 5 on chromosome 8.

The purpose of this study was to identify the MLPA results that correlate best with metastatic death from uveal melanoma so that estimation of the prognosis for survival can be enhanced.

Methods

Patients

DNA from frozen tumor specimens remaining from a previous study on uveal melanoma were retrieved from storage.[16] The patients had been

referred to the Liverpool Ocular Oncology Center between January 1998 and February 2000. They underwent full preoperative ophthalmic and systemic examinations that included slit lamp and ophthalmoscopic examination and echographic measurement of largest and smallest basal tumor dimensions and tumor thickness. Treatment was by enucleation or transscleral local resection. As soon as possible after surgery, a fresh tumor sample was collected for experimental purposes. Part of each tumor was snap frozen in liquid nitrogen, and the remainder was formalin fixed and embedded in paraffin. Histologic features were recorded from hematoxylin and eosin and PAS-stained sections of paraffin-embedded tumors.

Clinical, histologic, and chromosomal data were recorded in a computerized database. Patients residing in mainland Britain were flagged in the National Cancer Registry, which automatically informed us of any deaths, together with the date and cause of death. Follow-up information on overseas patients was obtained whenever possible by writing to the referring ophthalmologist or family doctor. The time until death was calculated from the date of local resection or enucleation.

The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from the patients after explanation of the nature and possible consequences of the study. Ethics committee approval was obtained.

MLPA Technique

DNA extraction, DNA quality assessment and quantification, and MLPA were performed as previously reported but with an improved protocol (Dopierala J, et al., unpublished data, 2008).[15]

DNA Extraction

Briefly, the frozen uveal melanoma samples and control specimens consisting of normal choroidal tissue were collected in microfuge tubes. Tissue lysis and protein digestion were performed in a mix of 87% TSE buffer (10 mM Tris/HCl [pH 7.5], 0.4 M NaCl, 2 mM EDTA [pH 8.0]), 0.9% of SDS, and proteinase K (1.64 mg/μL; Qiagen GmbH, Hilden, Germany). Samples were incubated overnight at 56°C (Eppendorf Thermomixer Comfort; Sigma-Aldrich, St. Louis, MO). Fresh proteinase K was added after 16 hours of incubation, and samples were incubated for an additional 4 hours. Genomic DNA was isolated by using a high salt concentration (5 M NaCl) and ethanol precipitation. DNA was dissolved in 20 to 100 μL of TE buffer (10 mM Tris [pH 8.5], 1 mM EDTA [pH 8.0]), depending on pellet size.

DNA Quantification and Quality Assessment

DNA concentration and absorbance at 280 and 260 nm were measured (Nano Drop System; NanoDrop, Minneapolis, MN). FSH-receptor PCR (forward primer CTA CCC TGC ACA AAG ACA GTG; reverse primer GTG TAC GTC ATG TCA AAT CCT CTG C) was performed to assess DNA quality in a thermal cycler (G-Storm GS1; GRI, Ltd., Braintree, UK), with a DNA polymerase kit (Go*Taq* Flexi; Promega, Madison WI). FSH-receptor PCR products were run on stained 2% agarose gel (SYBR Safe stain; Invitrogen; Groningen, The Netherlands). Electrophoresis was run at 120 V for 30 minutes, and the results were visualized with a transilluminator (Benchtop 2UV; Bio Doc-It Imaging System; UVP, Upland, CA).

MLPA Procedure and Sequencing

An MLPA kit (Salsa P027; MRC-Holland) was used to identify chromosomal imbalances in the uveal melanoma samples. The kit consists of 31 probes selected on chromosomes 1 (short arm), 3, 6, and 8, which often show aberrant copy numbers in uveal melanoma cells, as well as 12 control probes on chromosomes 5, 7, 12, 14, 18, 19, 20, and 21. Two-hundred nanogram aliquots of DNA extracted from uveal melanoma cells as well as four to six normal choroidal tissue control samples were dissolved in 5 μL of TE buffer and transferred to PCR tubes. TE buffer (5 μL) was used as a negative control in each run. The MLPA reaction was performed in the thermal cycler (G-Storm GS1; GRI Ltd.). After the MLPA PCR reaction, 4 μL of these products were mixed with loading buffer and were run on a stained 2% agarose gel (SYBR Safe stain; Invitrogen) to confirm amplification of the ligation products. After amplification was confirmed, 1 μL of MLPA products was mixed with 8.8 μL of highly deionized HI-DI formamide (Applied Biosystems, Inc. [ABI], Foster City, CA) and 0.2 μL of an internal lane size standard (GeneScan-500 LIZ Size Standard; ABI). Samples were separated by electrophoresis on a 16-capillary genetic analyzer (model 3130XL, analyzed by GeneMapper software; ABI) in the Molecular Genetics Department of the Liverpool Women's Hospital. Data were received as peak heights, as a measure of peak intensity, for each of 43 probes for each sample. Each tumor sample was tested using MLPA at least three independent times.

Data Analysis

Raw data obtained after sequencing were analyzed in a spreadsheet-based approach (Excel; Microsoft, Redmond, WA) designed by the National Genetics Reference Laboratory, Manchester, UK (http://www.ngrl.org.uk/Manchester/mlpapubs.html). The results from the uveal melanoma samples were compared with a group of five normal choroid tissue controls, run simultaneously. Data from each test and control sample were normalized by summing the total control probes' peak height and by dividing each ligation product's peak height by this figure. The control and test data were then equalized by dividing the normalized peak height by mean peak height of all five controls. Linear regression was performed to correct for sloping. Regression coefficients were calculated for each of five controls and the test samples, according to the probe lengths. Equalized control and test sample's ligation products were corrected for sloping, and the average results from five control samples for each ligation product were calculated. The Dosage Quotients (DQs) were calculated by dividing the test sample matrix by the control mean matrix. The DQ was categorized as: low, <0.65; equivocally low, 0.65–0.84; normal, 0.85–1.14; equivocally high, 1.15–1.34; and high, >1.34. The standard deviation of mean DQs of control probes for normal control and test samples were calculated. Samples were included in this analysis only if the DQs of the control probes had an SD < 0.2 (higher values indicating poor DNA quality or DNA fragmentation). This amounted to a total of 73 uveal melanomas; 30 samples were excluded.

Correlations between MLPA results and survival were performed (ver.11.0; SPSS Science, Chicago, IL), and Kaplan-Meier estimates were used to draw survival curves for time to metastasis-related death. Associations between metastasis-related death and risk factors were assessed with log rank analysis for categorical variables and with Cox univariate analysis for MLPA DQs.

Results

The 73 patients (46 men and 27 women) had a mean age of 60.6 years (range, 25.2–90.5). The tumors had a mean LBD of 15.0 mm (range, 5.1–21.2), with 31 involving ciliary body (i.e., pars plana and/or pars plicata) and 2 extending extraocularly. Histologic examination showed epithelioid cells in 59 tumors and closed loops in 51 tumors, with the mitotic rate exceeding 5 per 40 high-power fields in 34 tumors. MLPA showed no chromosome 3 abnormalities in 31 patients, 1 abnormal locus in 4 patients, 2 to 9 abnormal loci in 12 patients, and 10 to 13 abnormal loci in 26. The follow-up times had a median of 6.2 years in 35 survivors, exceeding 5 years in 33 patients and 8 years in 31. A total of 38 patients died, the cause of death being metastatic uveal melanoma in 28, other malignancy in 2, and other causes in 8 (cardiac failure, 2; myocardial infarction, 2; cerebrovascular disease, 1; old age, 1; pneumonia, 1; and uncertain, 1). One patient developed hepatic metastasis after treatment of a monosomy 3 melanoma and was alive and well at the close of the study, having undergone a successful partial hepatectomy.

Nine patients had FISH analysis of their tumors, which showed monosomy 3 in four cases and disomy 3 in five. In the four tumors with monosomy 3 on FISH testing, MLPA showed unequivocal loss of 12 or 13 chromosome 3 loci in three cases and equivocal loss of eight loci in one. In the five tumors with disomy 3 on FISH testing, MLPA showed equivocal loss of one chromosome 3 locus in two tumors. Eight tumors had FISH data on chromosome 8. In the three tumors with no chromosome 8 gains on FISH testing, MLPA showed equivocal gain of one locus in one case and unequivocal gains in three loci in another. FISH indicated chromosome 8 gains in five cases, and in these MLPA showed unequivocal gains of all four 8q loci in four tumors and equivocal gains of three adjacent 8q loci in one. MLPA indicated the 8p locus to be normal in seven tumors and lost in one case of monosomy 3.

Table 1 shows the correlations between MLPA results and metastatic death, using Cox univariate analysis. All chromosome 3 loci (i.e., losses) and all chromosome 8q loci (i.e., gains) correlated strongly with metastatic death. Gains in 6p25 showed a strong inverse correlation with metastasis (i.e., strongly indicated a good prognosis). Loss of 8p showed a borderline correlation with worse prognosis. Figure 1 shows boxplots of the MLPA results according to survival.

Table 2 shows the results of Cox multivariate analysis including 3q12, 8q11.23, and 6p25.2 in the model predicting metastatic death. Only loci in chromosomes 3 and 8 showed significant correlations.

Figure 2 shows Kaplan-Meier survival curves according to whether chromosome 3 loss was considered abnormal if the MLPA results were equivocal (Fig. 2A) or unequivocal (Fig. 2B). Figure 2A indicates that (1) when only unequivocal losses of chromosome 3 loci were considered abnormal, 4 (13%) of 31 patients without such definite abnormalities died of metastatic disease; and (2) high mortality occurred irrespective of the number of abnormal chromosome 3 loci. In Figure 2B, when even equivocal losses of chromosome 3 loci were considered abnormal, only 1 (4.5%) of 22 patients with normal chromosome 3 results died of metastatic disease (this was an unusual tumor, see Fig. 3A). Mortality was higher if all 13 chromosome 3 loci examined using MLPA showed equivocal loss than if fewer loci were abnormal. There were 21 patients with equivocal losses involving only part of chromosome 3, and in these patients the 10-year survival probability was 60%. In Figure 2C, tumors were categorized as having no abnormalities in chromosome 3, only equivocal abnormality and definite abnormality (i.e., unequivocal loss of any locus). Only nine patients showed a moderate survival prognosis (a result that is clinically unhelpful). Figure 2D shows survival according to whether there was loss of any chromosome 3 loci and whether there was gain in 8q11.23. Such abnormalities were considered sig-

Table 1. Cox Univariate Analysis Correlating MLPA Results with Metastatic Death

							95% CI for Exp(B)	
Probe	**Locus**	**B**	**SE**	**Wald**	**Sig.**	**Exp(B)**	**Lower**	**Upper**
MFN2	1p36.22−	−1.017	0.980	1.076	0.300	0.362	0.053	2.470
NBL1	1p36.13−	−1.379	0.955	2.085	0.149	0.252	0.039	1.637
PTAFR	1p.34−	−0.606	0.934	0.421	0.517	0.546	0.088	3.402
MYCBP	1p.34−	−0.194	0.945	0.042	0.838	0.824	0.129	5.255
MUTYH	1p33−	−0.554	0.910	0.371	0.543	0.575	0.097	3.417
RPE65	1p31−	−1.457	1.081	1.818	0.178	0.233	0.028	1.937
NOTCH2	1p11.2−	−2.093	1.172	3.189	0.074	0.123	0.012	1.227
FANCD2(i)	3p25.3−	−4.042	1.104	13.417	<0.001	0.018	0.002	0.153
FANCD2(ii)	3p24.3−	−2.981	0.877	11.542	0.001	0.051	0.009	0.283
VHL	3p25.3−	−3.093	0.876	12.474	<0.001	0.045	0.008	0.252
MLH11	3p22.1−	−3.093	0.876	12.474	<0.001	0.045	0.008	0.252
CTNNB11	3p22−	−3.163	0.943	11.246	0.001	0.042	0.007	0.269
SEMA3B	3p21.3−	−4.105	1.086	14.283	<0.001	0.016	0.002	0.139
FHIT(i)	3p14.2−	−3.250	0.931	12.198	<0.001	0.039	0.006	0.240
FHIT(ii)	3p14.2−	−3.420	0.998	11.738	0.001	0.033	0.005	0.231
ROBO1	3p12.2−	−3.131	0.868	13.001	<0.001	0.044	0.008	0.240
CPO	3q12−	−4.774	1.175	16.495	<0.001	0.008	0.001	0.085
RHO	3q21.3−	−3.589	0.988	13.186	<0.001	0.028	0.004	0.192
MME	3q25.1−	−4.620	1.210	14.580	<0.001	0.010	0.001	0.106
OPA1	3q29−	−4.594	1.178	15.210	<0.001	0.010	0.001	0.102
FOXC1	6p25+	−1.874	0.692	7.326	0.007	0.153	0.039	0.596
SERPINB9	6p25.2+	−2.097	0.705	8.850	0.003	0.123	0.031	0.489
CDKN1A	6p21.2+	−1.497	0.732	4.175	0.041	0.224	0.053	0.941
RUNX2	6p21+	−1.964	0.868	5.121	0.024	0.140	0.026	0.769
CTGF	6q23.1−	−0.332	0.858	0.150	0.698	0.717	0.133	3.856
IGF2R	6q26−	−0.306	0.894	0.117	0.732	0.736	0.128	4.243
NRG1	8p12−	−1.822	0.883	4.260	0.039	0.162	0.029	0.912
RP1	8q11.23+	1.666	0.304	29.932	<0.001	5.291	2.913	9.609
MYC(i)	8q24.12+	1.415	0.337	17.674	<0.001	4.118	2.129	7.966
MYC(ii)	8q24.12+	1.330	0.328	16.433	<0.001	3.779	1.987	7.188
DDEF1	8q24.2+	1.500	0.328	20.861	<0.001	4.480	2.354	8.527

MFN2, mitofusin2; *NBL1,* neuroblastoma, suppressor of tumorigenicity 1; *PTAFR,* platelet activating factor receptor; *MCYBP,* c-Myc binding protein; *MUTYH,* mutY homolog; RPE65 retinal pigment epithelium specific protein 65; *NOTCH2,* notch homolog 2; *FANCD2,* Fanconi anemia complementation group D2; *VHL,* von Hippel Lindau; *MLH1,* MutL homolog 1; *CTNNB1,* catenin, beta-1; *SEMA3B,* semaphoring 3B; *FHIT,* fragile histidine triad gene; *ROBO1,* roundabout, axon guidance receptor, homolog 1; *CPO,* coproporphyrinogen oxidase; *RHO,* rhodopsin; *MME,* membrane metallo-endopeptidase; *OPA1,* optic atrophy 1; *FOXC1,* forkhead box Cl; *SERPINB9,* serpin peptidase inhibitor, clade B, member 9; *CDKN1A,* cyclin dependent kinase inhibitor 1A; *RUNX2,* runt related transcription factor; *CTGF,* connective tissue growth factor; *IGF2R,* insulin-like growth factor 2 receptor; *NRG1,* neuregulin 1; *RP1,* retinitis pigmentosa 1; *DDEF1, DDEF* intronic transcript 1; B, B statistic; Sig., significance; Exp, exponent; CI, confidence interval.

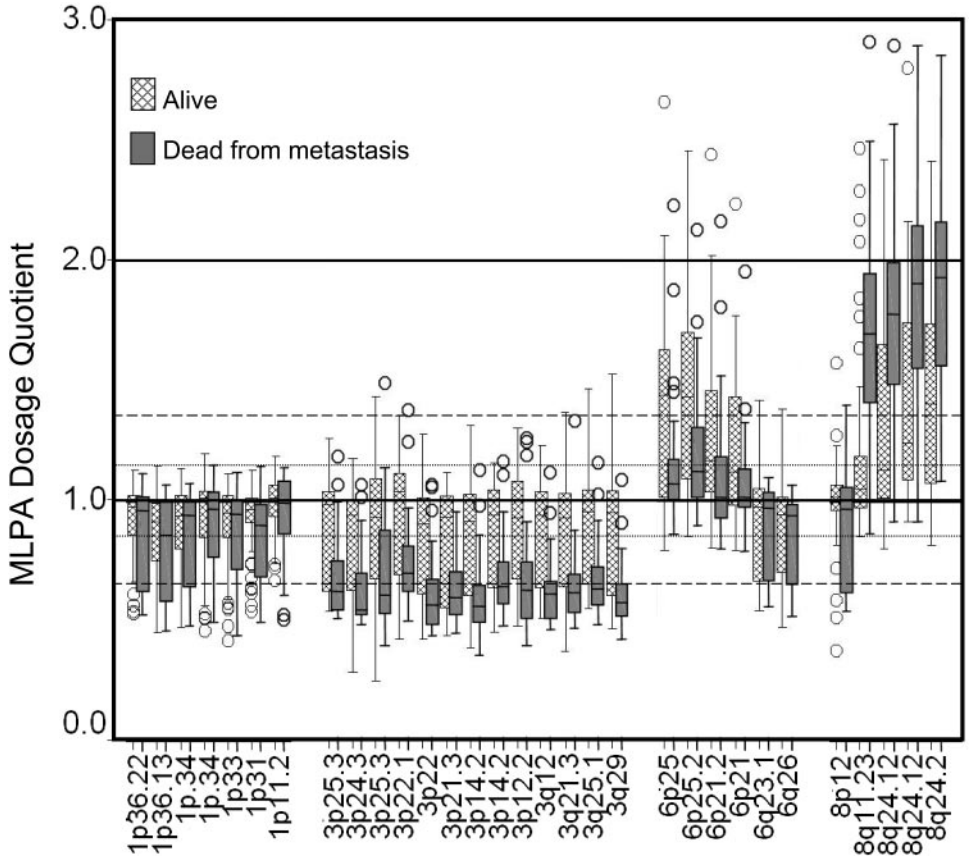

FIGURE 1. Dosage quotients of each MLPA probe, according to survival, with nonfatal and fatal tumors indicated by *hatched* and *gray boxes*, respectively. *Dotted lines*: separate normal from equivocal abnormality; *dashed lines*: demarcate equivocal from definite abnormality.

nificant, even if equivocal. None of six patients with no abnormalities died of metastasis compared with 80% of patients with abnormality of both chromosomes.

Figure 3A shows the MLPA results of the above-mentioned single patient with no chromosome 3 loss (i.e., equivocal or unequivocal). This patient showed apparent gains in 3p25.3 and 3p22.1 and low normal values of 3p21.3 and 3q21.3 as well as gains in 8q. Three patients showed equivocal loss of one or more chromosome 3 loci (Figs. 3B–D). The patient in Figure 3B showed equivocal loss of all four 3q loci with equivocal gains in 8q. The patient in Figure 3C showed equivocal losses of several 3p loci and 3q29 and gains in 8q. Case 3D showed equivocal losses in 3p22 and 3p14.2 as well as gains in 6p and 8q.

Figure 4 shows survival in patients with lethal melanomas (i.e., tumors with equivocal or definite abnormality of chromosomes 3 and/or 8q). Survival correlated strongly with tumor diameter (log rank, $P = 0.002$; Fig. 4A) and presence of closed loops (log rank, $P = 0.002$; Fig. 4C), weakly with the presence of epithelioid cells (probably because few lethal melanomas were purely of spindle cell type) (log rank, $P = 0.024$) (Fig. 4B), and nonsignificantly with mitotic rate (log rank, $P = 0.14$; Fig. 4D).

DISCUSSION

To our knowledge, this is the first study to correlate metastatic death with MLPA results obtained with the Salsa P027 uveal melanoma kit (MRC-Holland). We found that several fatal tumors showed only equivocal MLPA abnormalities of chromosome 3 loci. Correlation with metastatic disease improved when we considered other risk factors, such as chromosome 8 gains, basal tumor diameter, and histologic features suggestive of high-grade malignancy, such as cell type, and closed loops 1p and 6p abnormalities were relatively uninformative.

We selected MLPA to examine the chromosomal abnormalities in uveal melanomas because it provides more information than FISH, the sensitivity of which has been questioned by us and others.[11,17] MLPA also requires smaller samples than FISH and can be used on DNA extracted from formalin-fixed, paraffin-embedded tissues.[15] Furthermore, it is relatively inexpensive, so that it is affordable for routine clinical use in a large number of patients. MLPA has been used to investigate uveal melanomas (deploying an aneuploidy kit, Salsa P095; MRC-Holland) and cutaneous melanocytic tumors.[18,19] We are currently using MLPA as the first-line investigational procedure for chromosomal abnormalities in uveal melanomas recently diag-

TABLE 2. Cox Multivariate Analysis Correlating MLPA Results with Metastatic Death

						95% CI for Exp(B)	
Locus	**B**	**SE**	**Wald**	**Sig.**	**Exp(B)**	**Lower**	**Upper**
3q12−	−4.085	1.525	7.176	0.007	0.017	0.001	0.334
8q11.23+	1.220	0.339	12.980	<0.001	3.388	1.745	6.581
6p25.2+	0.292	0.833	0.123	0.726	1.339	0.262	6.852

B, B statistic; Sig., significance; Exp, exponent; CI, confidence interval.

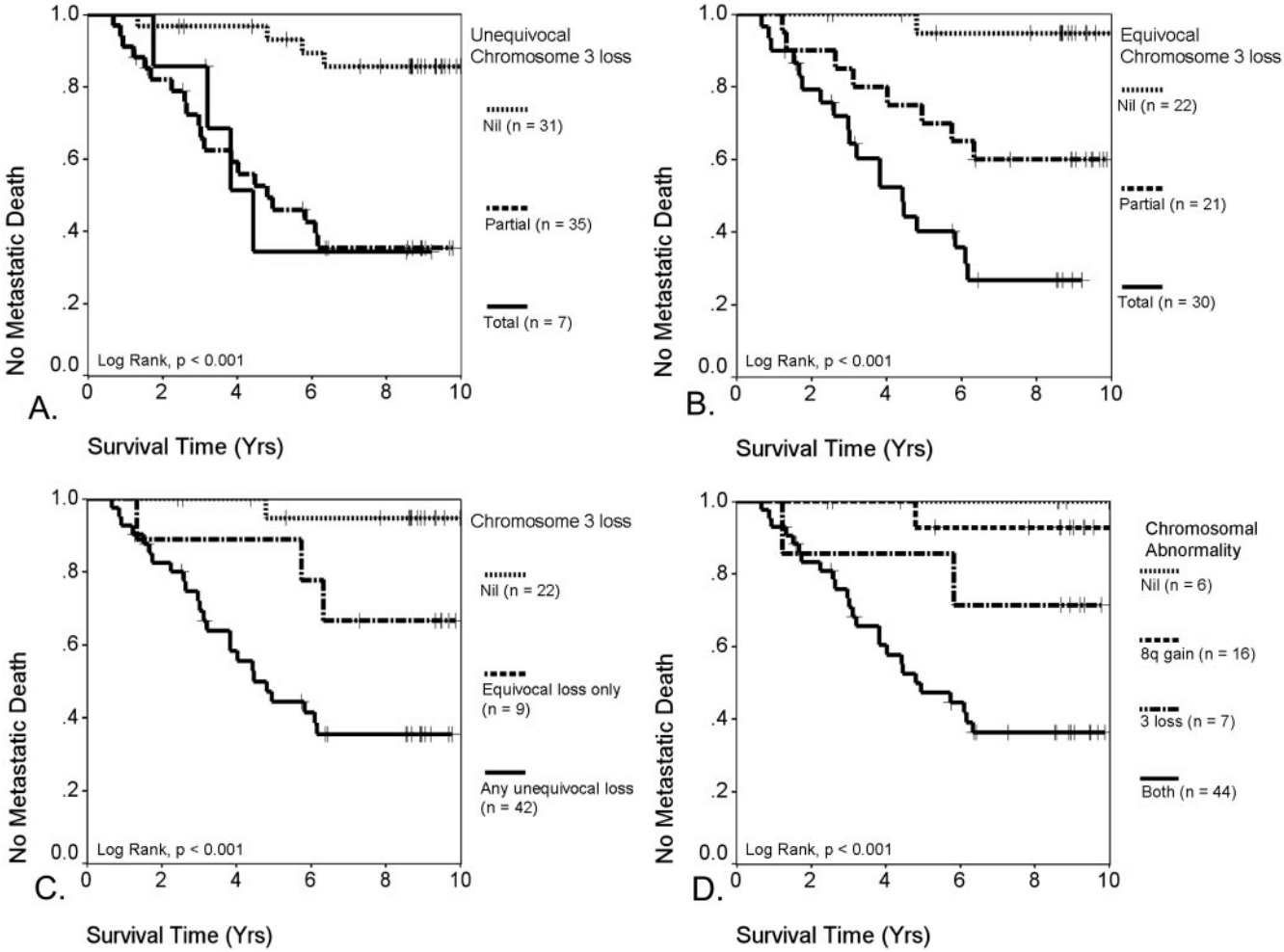

FIGURE 2. Kaplan-Meier curves showing rates of metastatic death according to (**A**) unequivocal (i.e., definite) losses in some or all chromosome 3 loci; (**B**) equivocal losses in some or all chromosome 3 loci; (**C**) equivocal and definite losses in chromosome 3; and (**D**) equivocal or definite abnormality of any chromosome 3 locus and/or 8q11.23.

nosed at the Liverpool Ocular Oncology Center and have analyzed more than 250 tumors with this method. With small biopsy samples, MLPA often succeeded when FISH failed, whereas the converse only rarely occurred (Damato B, et al., unpublished data, 2009).

Despite including 73 samples, the present study is weakened by the relatively small number of patients. In addition, the median follow-up of 6.2 years was probably not long enough to identify all patients destined to die of metastatic disease. Furthermore, several patients died of unrelated causes, possibly masking metastatic deaths. Another limitation is that postmortem examinations were not performed, so that there is uncertainty about the exact cause of death in some patients. Some overseas patients were lost to follow-up and may have died of metastasis. Finally, the survival data may have been influenced, albeit slightly, by apparently successful resection of liver metastasis in one patient.

It was not a purpose of this study to compare the MLPA results with those of FISH, which we started performing only toward the end of the period when these patients were treated. MLPA confirmed all abnormal FISH results. In addition, MLPA revealed equivocal abnormalities in chromosomes 3 and 8 and unequivocal gains in chromosome 8 that were not detected with FISH. We are presently conducting a more in-depth comparison of MLPA with FISH in a large cohort of patients treated since 2006. We are also comparing MLPA with microsatellite analysis (MSA) and will publish the results in another article. Briefly, MLPA detected equivocal and unequivocal chromosome 3 abnormalities in 7 and 10 of 32 tumors showing no loss of heterozygosity (LOH) with MSA. Conversely, all 41 tumors with LOH on MSA showed abnormalities with MLPA (Damato B, et al., unpublished data, 2008).

The results shown in Figure 2B suggest that equivocal losses in chromosome 3 using MLPA are associated with metastatic death, whether these involve part or all of the chromosome. It must be emphasized that only uveal melanoma samples with good quality MLPA data (i.e., control probes having SD < 0.2) were included for interpretation in this study. If only definite chromosome 3 losses were considered abnormal (i.e., a DQ of <0.65), then more than 10% of patients with a so-called normal MLPA result would have died of metastatic disease. Such a high false-negative rate would be unacceptable in the clinical arena, essentially preventing any patients from receiving reassurance about a good prognosis. If even equivocal chromosome 3 losses were considered abnormal (i.e., a DQ between 0.65 and 0.84), then this false-negative rate would be reduced, to the extent that patients could be confidently reassured of a good prognosis. Categorization of tumors according to the number of chromosome 3 loci with equivocal loss resulted, however, in a high proportion of patients with a 60% 10-year survival probability. Such an uncertain prognosis is not clinically useful because, on the one hand, it does not provide reassurance to

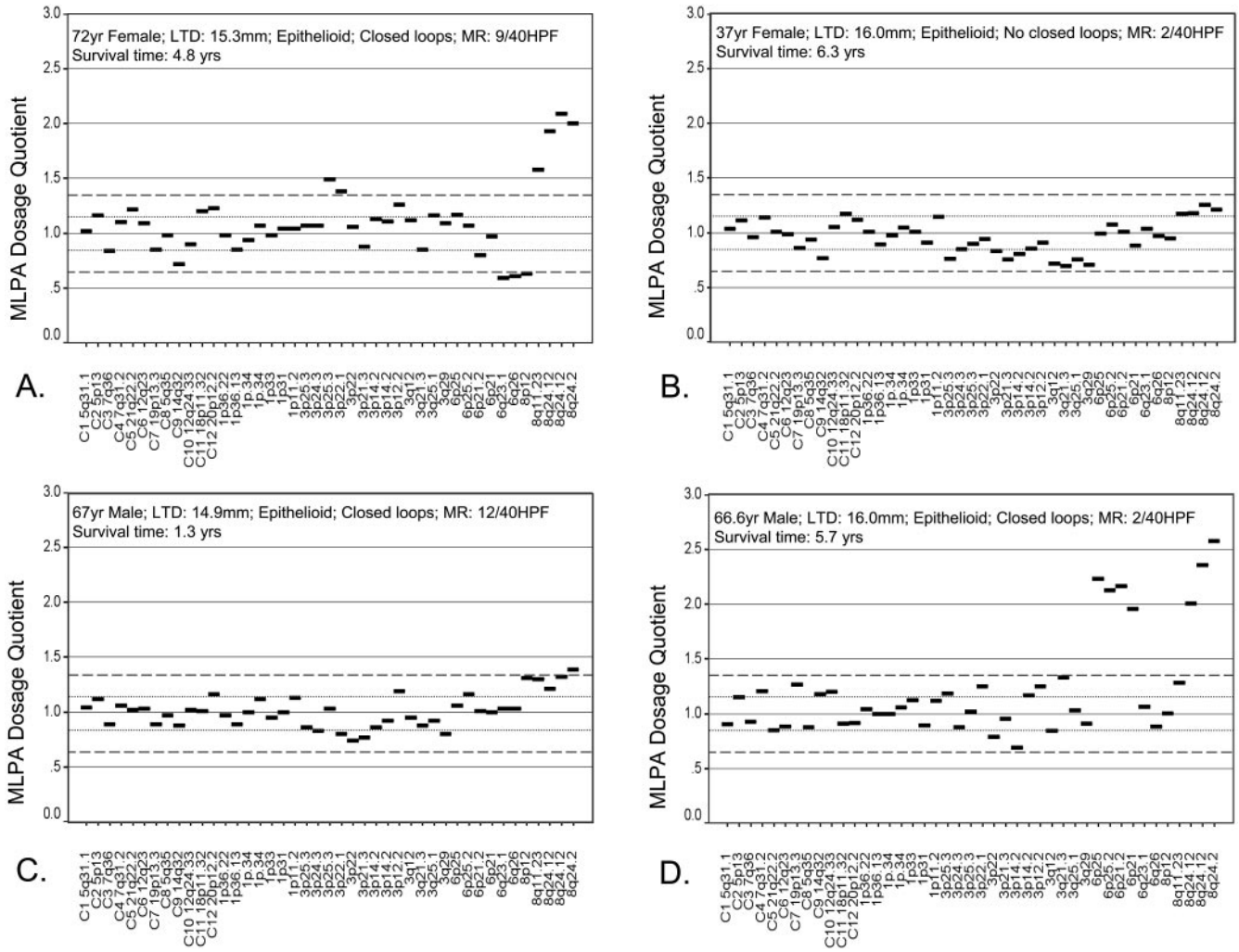

FIGURE 3. MLPA results of four patients, who died of metastatic disease, but who had uveal melanomas with no definite chromosome 3 losses. (**A**) Gains in chromosome 3p (VHL and MLH1) loci and low normal chromosome 3p and 3q (SEMA3B and RHO) values. (**B**) Equivocal losses of all 3q loci. (**C**, **D**) Equivocal losses on the long and short arms of chromosome 3. The DNA of (**D**) was of inferior quality as shown by the control values. Nevertheless, this tumor was unusual in that it showed gains in 6p together with partial and equivocal deletions of 3p. *Dotted lines*: separate normal from equivocal abnormality; *dashed lines*: demarcate equivocal from definite abnormality.

patients and, on the other hand, it does not identify high-risk patients requiring special care, such as inclusion in any clinical trials of systemic adjuvant therapy. Categorization of patients into distinct low-risk, and high-risk groups was improved by grading tumors according to the presence of equivocal or definite chromosome 3 losses (Fig. 2C). Prognostication was also improved by considering chromosome 3 losses together with 8q gains (Fig. 2D).

The results of this study are consistent with the findings in our previous studies with regards to chromosome 3 and 8 abnormalities and tumor diameter.[11,14] Tumor cell type was relatively unimportant, however, probably because almost all lethal uveal melanomas contained epithelioid cells in varying proportions. As in previous studies, 6p gain correlated with a good survival prognosis; however, it was excluded as an independent predictive factor by multivariate analysis. The detection of 6p gain is nevertheless useful because its presence confirms that the specimen contains tumor cells.

The most likely explanation for equivocal MLPA results (i.e., a DQ between 0.65 and 0.84) is cellular heterogeneity, with disomy 3 cells diluting measurements from monosomy 3 cells. Other studies have demonstrated clonal and genetic heterogeneity in uveal melanoma.[20–22] The coexistence of 6p gains and chromosome 3 losses, described to occur in approximately 4% of uveal melanomas, also merits further investigation.[23] The dilution effect of such cellular heterogeneity makes it necessary for equivocal MLPA results to be interpreted together with additional tests, such as fluorescence in situ hybridization, so as to minimize any chances of missing monosomy 3. Because MLPA provides only relative and not absolute values, FISH is also useful as a means of distinguishing monosomy 3 from disomy 3 with hyperploidy. It is likely that the problems caused by cellular heterogeneity will complicate the interpretation of methods other than MLPA, such as single nucleotide polymorphism (SNP) arrays.

Our results suggest that there is a possibility of improving the MLPA kit for uveal melanoma, possibly adding loci on chromosome 3 as well as on 8p. The criteria for abnormality also should be revised, to take account of cellular heterogeneity. A further limitation of MLPA is that results may be influenced by abnormalities in genomic loci used as controls. As tumor cells have an unstable genome, additional positive internal controls may have to be considered in MLPA investigations.

Further studies are needed to validate the findings of this investigation, using a new cohort of patients. Such studies

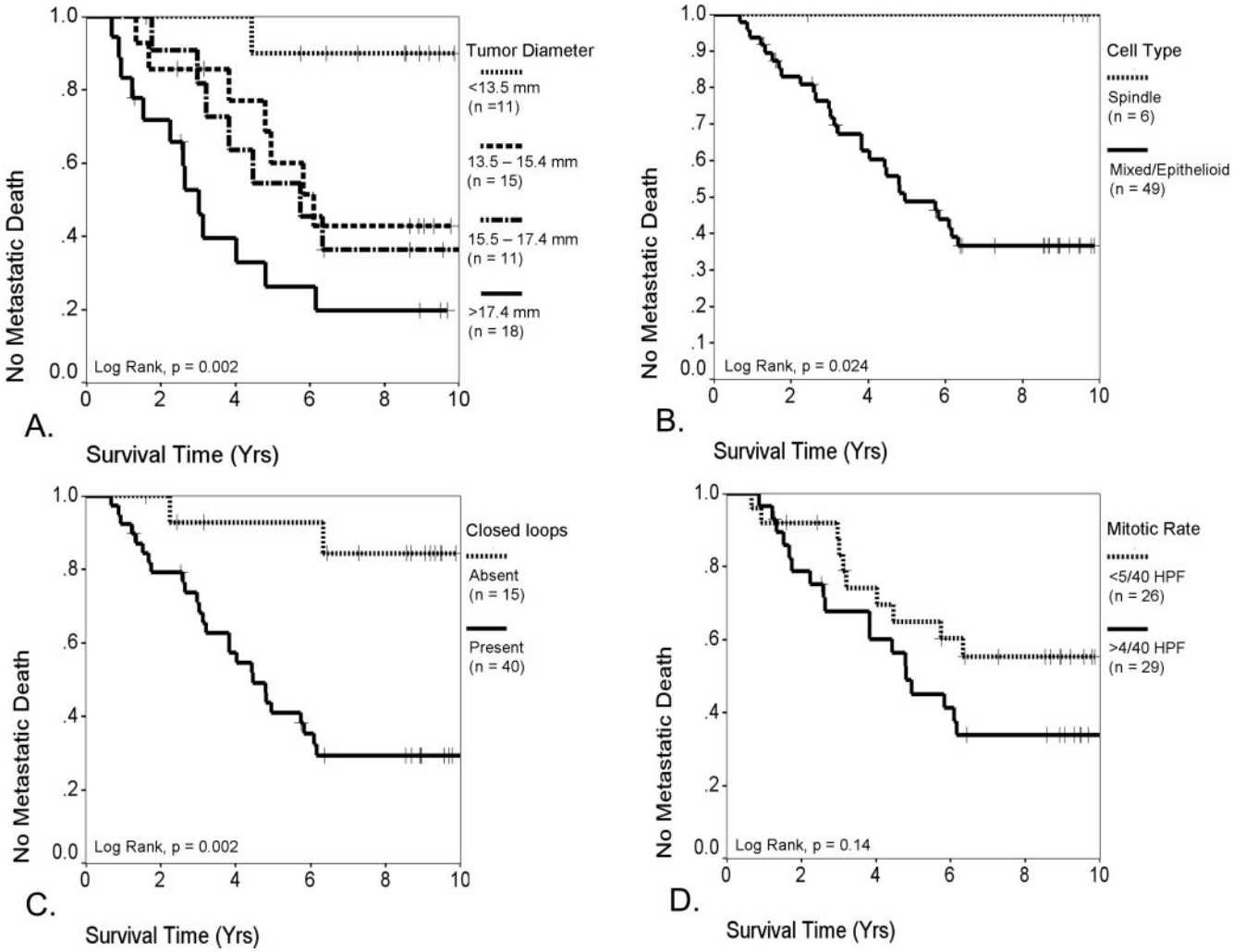

Figure 4. Kaplan-Meier survival curves showing rates of metastatic death in patients with lethal uveal melanoma (i.e., equivocal or definite loss of any chromosome 3 loci), according to (**A**) basal tumor diameter, (**B**) presence of epithelioid melanoma cells, (**C**) closed laminin loops, and (**D**) mitotic rate.

would enable estimation of sensitivity and specificity of MLPA in predicting metastatic death, as well as positive and negative predictive values. It is also necessary to investigate specifically the predictive value of MLPA when performed on tiny tumor specimens, obtained by fine needle aspiration biopsy or with a vitreous cutter. This is because in mosaic tumors, such tumor sampling may miss the most aggressive parts of the tumor.

MLPA and other methods of genomic tumor typing only determine whether the uveal melanoma has any metastatic potential. If the tumor is indeed lethal, then to estimate the likely survival time, it is necessary to take account of (1) clinical tumor stage, which indicates how long any metastases are likely to have been growing (i.e., lead-time bias); and (2) histologic grade, which reflects the rate of growth of such metastases (i.e., tumor doubling time).

In conclusion, MLPA is useful for estimating the probability of metastatic death after treatment of uveal melanoma, but requires multivariate analysis of chromosomes 3 and 8, also taking into account the clinical stage and histologic grade of tumor malignancy.

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